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APPLICATION NUMBER: ***60/537,018***
FILING DATE: ***January 20, 2004***

PRIORITY DOCUMENT
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T. LAWRENCE
Certifying Officer

PATENT APPLICATION SERIAL NO. _____

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PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

01/21/2004 EFLORES 00000108 60537018
01 FC:2005 80.00 OP

PTO-1556
(5/87)

*U.S. Government Printing Office: 2002 — 489-267/69033

PTO/SB/16 (08-03)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

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☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

METHODS FOR DETECTING AND TREATING CANCER

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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OR

☐ Firm or Individual Name					
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Address					
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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 47 ☐ CD(s), Number

☒ Drawing(s) *Number of Sheets* 5 ☐ Other (specify)

☒ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees

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[Page 1 of 1]

Respectfully submitted,

SIGNATURE M. Gravelle Date JAN. 16, 2004

TYPED or PRINTED NAME MICHELINE GRAVELLE REGISTRATION NO. *(if appropriate)* 40,261

TELEPHONE 416-957-1682 Docket Number: 7685-77

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PTO/SB/17 (10-03)
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FEE TRANSMITTAL for FY 2004

Effective 10/01/2003. Patent fees are subject to annual revision.

☑ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
Application Number	
Filing Date	
First Named Inventor	KELLY MCNAGNY
Examiner Name	
Art Unit	
Attorney Docket No.	7685-77

METHOD OF PAYMENT (check all that apply)

☑ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☑ Deposit Account: #6200

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FEE CALCULATION

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Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra Claims		Fee from below	Fee Paid
Total Claims	- 20** =		X	= 0.00
Independent Claims	- 3* =		X	= 0.00
Multiple Dependent				=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0.00

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
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1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($) 0.00

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Patent Application Data She t

Application Information

Application Type::	Provisional
Subject Matter::	Utility
Suggested Classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	None
Number of CD disks::	0
Number of copies of CDs::	0
Sequence submission?::	NO
Computer Readable Form (CRF)?::	NO
Number of copies of CRF::	0
Title::	METHODS FOR DETECTING AND TREATING CANCER
Attorney Docket Number::	7685-77
Request for Early Publication?::	NO
Request for Non-Publication?::	NO
Suggested Drawing Figure::	
Total Drawing Sheets::	5
Small Entity?::	Yes
Latin Name::	
Variety denomination name::	

Initial 1/16/04

Petition included?::	No
Petition Type::	
Licensed US Govt. Agency::	
Contract or Grant Numbers::	
Secrecy Order in Parent Appl.?::	No

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Representative Information

Representative Customer Number::	**001059**

Domestic Priority Information

Initial 1/16/04

Application::	Continuity Type::	Parent Application::	Parent Filing Date::

Foreign Priority Applications

Country::	Application Number::	Filing Date::	Priority Claimed

Initial 1/16/04

B&P File No. 7685-77/MG/MS

BERESKIN & PARR

UNITED STATES PROVISIONAL

Title: METHODS FOR DETECTING AND TREATING CANCER
Inventors: Kelly M. McNagny, Calvin Roskelley and Helen Merkens

TITLE: Methods For Detecting And Treating Cancer

FIELD OF THE INVENTION

The invention relates to methods and kits for detecting and monitoring the progression of cancer, in particular breast cancer. The invention also includes methods of treating cancer.

BACKGROUND OF THE INVENTION

Metastatic breast cancer is the leading cause of death among women between the ages of 15 and 54 and affects approximately 13% of women during their lifespan. These can be grossly categorized as ductal or lobular depending on their site of origin in normal breast tissue. Tumors usually begin as non-invasive cells at the site of tumor origin, spread to surrounding tissue in the breast and eventually become fully metastatic and migrate to the lymph nodes and other parts of the body. There is increasing evidence that cell-cell adhesion is a potent suppressor of metastatic breast cancer progression (Berx and Van Roy, 2001).

CD34 was initially identified over 20 years ago as an hematopoietic stem cell and vascular endothelial marker and has alternatively been proposed to act as an: 1) enhancer of proliferation, 2) a blocker of differentiation, 3) bone marrow homing receptor, 4) cell adhesion molecule, and 5) a blocker of cell adhesion (Fackler et al, 1996, Krause et al. Blood, 1996, Baumhueter et al. 1993). Deletion of the CD34 gene in mice has only served to fuel this debate as these mice are relatively normal with very subtle defects in hematopoietic and vascular function. The most clear-cut experiments suggest that CD34-type proteins can act as either pro-adhesive or anti-adhesive molecules depending on their glycosylation status (Satomaa, 2002, Baumhueter et al., 1993 and Bistrup et al., 1999).

Podocalyxin, (also called podocalyxin-like protein 1 (PCLP-1), Myb-Ets-transformed progenitor (MEP21) or thrombomucin) is a heavily sialylated and sulfated integral membrane glycoprotein that interacts with the actin cytoskeleton. It belongs to the CD34 family of sialomucins and is highly expressed on the surface of hematopoeitic progenitors, vascular endothelia and podocytes which form a tight junction-free epithelial meshwork that

surrounds glomerular capillaries in the kidney (Kerjaschki et al., 1984; Kershaw et al., 1995; McNagny et al., 1997). Evidence suggests that the primary function of this molecule is to act as a type of molecular "Teflon™" to block inappropriate cell adhesion.

Circumstantial evidence suggests that podocalyxin expression may be upregulated in a variety of neoplastic scenarios. For example podocalyxin was recently identified as the peanut agglutinin-binding tumor antigen gp200 expressed on human embryonal carcinomas. (Schopperle et al., 2002). In addition, the human podocalyxin gene (PODXL) has been assigned to chromosome 7q32-q33 (Kershaw et al., 1997), which places PODXL very close to the 7q35ter region that has been identified as a gain site by comparative genomic hybridization in ductal carcinoma in situ, infiltrating ductal carcinoma and in lymph node metastasis (Aubele et al., 2000). Thus, while it is not yet clear whether the PODXL gene is amplified in breast carcinoma, its expression may be unduly influenced by a nearby amplicon. Under anemic conditions the inventors have recently shown that Podocalyxin expression is upregulated in mouse erythroid progenitor cells (McNagny submitted unpublished obs). Therefore, podocalyxin expression may be similarly upregulated in necrotic breast carcinomas where hypoxia-regulated genes are transcriptionally activated (Adeyinka et al., 2002). If this is indeed the case, it would have functionally important implications as tumor hypoxia helps to drive solid tumor progression generally (Knowles and Harris, 2001) and ductal carcinoma progression specifically (Bos et al., 2003; Helczynska et al., 2003).

Using homologies present in the cytoplasmic tails of CD34 and podocalyxin, endoglycan was identified as a novel member of this family of glycoproteins. Endoglycan mRNA and protein were detected in both endothelial cells and CD34+ bone marrow cells (Sassetti et al., 2000). Endoglycan, like CD34 and podocalyxin can function as a L-selectin ligand. Endoglycan utilizes a different binding mechanism, interacting with L-selectin through sulfation on two tyrosine residues and O-linked sLex structures (Fieger et al., 2003).

SUMMARY OF THE INVENTION

The present inventors have shown that endoglycan is an antagonist of podocalyxin, a novel prognostic indicator of tumor metastasis (see co-pending application of McNagny et al., application number 60/476,622).

Accordingly, in one embodiment, the present invention provides a method for detecting cancer in a patient comprising:

(a) obtaining a sample from the patient;

(b) determining the level of endoglycan in the sample; and

(c) comparing the level of endoglycan in the sample to a control sample, wherein decreased levels of endoglycan as compared to the control indicates that the patient has cancer.

In a further embodiment, the present invention provides a method for detecting cancer in a patient comprising:

(a) obtaining a sample from the patient;

(b) determining the level of endoglycan and podocalyxin in the sample; and

(c) comparing the ratio of endoglycan to podocalyxin in the sample to a control sample, wherein a decreased ratio of endoglycan to podocalyxin as compared to the control indicates that the patient has cancer.

In another embodiment, the present invention provides a method for monitoring the progression of cancer in a patient, comprising:

(a) obtaining a sample from a patient;

(b) determining the level of endoglycan in the sample;

(c) repeating steps (a) and (b) at a later point in time and comparing the result of step (b) with the result of step (c) wherein a difference in the level of endoglycan expression is indicative of the progression of the cancer in the patient.

In a further embodiment, the present invention provides a method for monitoring the progression of cancer in a patient comprising:

(a) obtaining a sample from a patient;

(b) determining the level of endoglycan and podocalyxin in the sample;

(c) repeating steps (a) and (b) at a later point in time and comparing the result of step (b) with the result of step (c) wherein a difference in the ratio of endoglycan to podocalyxin is indicative of the progression of the cancer in the patient.

In another embodiment, the present invention provides a method for determining whether or not a cancer is metastatic in a patient comprising:

(a) obtaining a sample from the patient;

(b) detecting the level of endoglycan in the sample; and

(c) comparing the level of endoglycan in the sample to a control sample, wherein decreased levels of endoglycan as compared to the control indicates that the cancer is metastatic.

In a further embodiment, the present invention provides a method for determining whether or not a cancer is metastatic in a patient comprising:

(a) obtaining a sample from a patient;

(b) detecting the level of endoglycan and podocalyxin in the sample; and

(c) comparing the ratio of endoglycan to podocalyxin in the sample to a control sample, wherein a decreased ratio of endoglycan to podocalyxin as compared to the control indicates that the cancer is metastatic.

In preferred embodiments of the invention, the above methods are used to detect breast cancer.

The present invention includes methods of treating cancer by modulating, preferably agonizing, the levels of endoglycan on the cancer. The application also includes methods for the identification of compounds that modulate the biological activity of endoglycan that may be used for the treatment of cancers with decreased expression of endoglycan.

Accordingly, the present invention provides a method of modulating cancer cell growth by administering an effective amount of an agent that modulates endoglycan to a cell or animal in need thereof.

The present invention also includes screening assays for identifying agents that modulate endoglycan and that are useful in modulating cancer cell growth. Agents that modulate include agents that stimulate endoglycan

(endoglycan agonists) and agents that inhibit endoglycan (endoglycan antagonists).

Accordingly, the present invention provides a method for identifying a compound that modulates endoglycan comprising:

(a) incubating a test compound with endoglycan or a nucleic acid encoding endoglycan; and

(b) determining the effect of the compound on endoglycan activity or expression and comparing with a control (i.e. in the absence of the test substance), wherein a change in the endoglycan activity or expression as compared to the control indicates that the test compound modulates endoglycan.

The present invention includes pharmaceutical compositions containing one or more modulators of endoglycan. Accordingly, the present invention provides a pharmaceutical composition for use in modulating cancer cell growth comprising an effective amount of endoglycan modulator in admixture with a suitable diluent or carrier.

In one embodiment, the present invention provides a pharmaceutical composition for use in treating cancer comprising an effective amount of an endoglycan agonist in admixture with a suitable diluent or carrier.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 shows the CD34 family including their genomic loci, motifs and splicing. **(A)** Schematic showing the hypothetical structure of CD34, Podocalyxin, and Endoglycan based on predicted protein sequences and

published data. Blue boxes = mucin domains, green boxes = the cysteine-rich domains, black circles = potential N-linked carbohydrates, horizontal bars with or without arrows = potential O-linked carbohydrates, arrows = potential sialic acid motifs on O-linked carbohydrates, PKC, CK2 and TK = potential phosphorylation sites. **(B)** Genomic organization of human *cd34*, *podxl* and *endgl* genes based on sequence contigs identified in the human sequence database. **(C)** Alternative splicing of CD34-family transcripts and their consequences for protein structure. Analyses of ESTs, primary cDNA clones and genomic loci suggest that, for all three family members, splicing between exons 7 and 8 results in longer cDNAs with premature translational stops that lead to truncation of the cytoplasmic domains.

Figure 2 shows homologies between CD34 family orthologs and homologs.

Figure 3 shows the specificity of rat monoclonal antibody F4B10 to endoglycan compared to other CD34 family members.

Figure 4 shows reciprocal expression of Endoglycan and Podocalyxin by metastatic and non-metastatic breast carcinoma lines. FACS profiles showing Endoglycan and Podocalyxin expression by the metastatic, non-polarized cell, MDA-231 and the non-metastatic, polarized cell line MCF-7. Green lines = specific antibody staining. Below is a western blot to show relative levels of Podocalyxin in these lines. MCF-7 and a second non-metastatic line (T47D) express high levels of Endoglycan but little if any Podocalyxin. MDA-231, a metastatic line expresses high levels of Podocalyxin and no Endoglycan.

Figure 5 shows failure of ectopic Endoglycan expression to block mast cell aggregation. **(A)** Mast cells from Wild type and $cd34^{-/-}/cd43^{-/-}$ infected with pMXpie retrovirus alone were plated at similar densities for assessment of aggregation. Graphs show data from two independently derived bone marrow mast cell cultures. **(B)** $cd34^{-/-}/cd43^{-/-}$ mast cells infected with pMXpie containing $CD34^{FL}$, $CD34^{CT}$ or Endoglycan. Graphs show data from two independent infections.

DETAILED DESCRIPTION OF THE INVENTION

I. Diagnostic Methods

The present inventors have determined that endoglycan and podocalyxin have a mirror image pattern of expression in breast cancer cells lines. Endoglycan levels are high in the relatively non-metastatic lines MCF-7 and T47D where podocalyxin levels are low. In contrast, endoglycan expression is negative in the MDA-231 metastatic tumor line compared to high levels of podocalyxin. Since endoglycan and podocalyxin have similar sequences in the cytoplasmic domain, endoglycan may be a natural antagonist of podocalyxin. Endoglycan may promote adhesion, maintain cell polarity and block metastasis whereas podocalyxin may block adhesion, decrease polarity and increase metastasis. Despite endoglycan's similarity to CD34 and podocalyxin (Figures 1 and 2), it does not block cell aggregation when ectopically expressed in CD34/CD43 deficient mast cells, a phenotype of ectopic expression of CD34. Podocalyxin is known to bind to the actin cytoskeleton through binding to NHERF (Li and Kershaw 2003, and Takeda 2002). Since endoglycan binds NHERF but lacks an anti-adhesive function, it may act as an antagonist of podocalyxin by competing with podocalyxin's ability to interact with the actin cytoskeleton and more specifically with NHERF.

Accordingly, evaluating endoglycan levels may be used in the prognostic and diagnostic evaluation of cancers involving endoglycan, the identification of subjects with a predisposition to such cancers, and in the monitoring of the progress of patients with endoglycan related cancers.

In an embodiment of the invention, a method is provided for detecting cancer in a patient comprising:

(a) obtaining a sample from the patient;

(b) detecting the level of endoglycan in the sample; and

(c) comparing the level of endoglycan in the sample to a control sample, wherein decreased levels of endoglycan as compared to the control indicates that the patient has cancer.

Evaluating endoglycan levels relative to podocalyxin levels may also be used in the prognostic and diagnostic evaluation of cancers involving endoglycan, the identification of subjects with a predisposition to such cancers, and in the monitoring of the progress of patients with endoglycan related cancers.

Accordingly, in another embodiment of the invention, a method is provided for detecting cancer in a patient comprising:

(a) obtaining a sample from the patient;

(b) determining the level of endoglycan and podocalyxin in the sample; and

(c) comparing the ration of endoglycan to podocalyxin in the sample to a control sample, wherein a decreased ratio as compared to control indicates that the patient has cancer.

The term endoglycan includes all of the known endoglycan molecules including those deposited in GenBank under accession number AF219137 or those referred to in Sassetti et al. (Sassetti C, Van Zante A, and SD Rosen, (2000) Identification of Endoglycan, a Member of the CD34/Podocalyxin Family of Sialomucins, Journal of Biological Chemistry, 275(12):9001) as well as any isoforms, variants, analogs, derivatives or fragments thereof that are useful in detecting cancer.

The term "podocalyxin" as used herein is synonymous with podocalyxin-like protein 1 (PCLP-1), Myb-Ets-transformed progenitor (MEP21) or thrombomucin and is a heavily sialyated and sulfated integral membrane glycoprotein that interacts with the actin cytoskeleton. The term podocalyxin includes all of the known podocalyxin molecules including those deposited in GenBank under accession number U97519 or those referred to in Kershaw et al. (Kershaw DB, Beck SG, Wharram BL, Wiggins JE, Goyal M, Thomas PE, Wiggins RC., Molecular cloning and characterization of human podocalyxin-like protein. Orthologous relationship to rabbit PCLP1 and rat podocalyxin. J Biol Chem. 1997 Jun 20;272(25):15708-14) as well as any isoforms, variants, analogs, derivatives or fragments thereof that are useful in detecting cancer.

The phrase "detecting the level of endoglycan" and "detecting the level of podocalyxin" includes the detection of the levels of protein as well as detection of the levels of nucleic acid molecules encoding the protein. Methods for detecting proteins and nucleic acids are discussed in greater detail below.

Endoglycan and podocalyxin are alternatively spliced to give two isoforms of the protein core; one with a long cytoplasmic tail and one with a short cytoplasmic tail. Consequently, in a specific embodiment, the methods of the invention are used to detect the short form of endoglycan (and podocalyxin).

The term "cancer" as used herein includes all cancers that are associated with decreased expression of endoglycan. In a preferred embodiment, the cancer is breast cancer, more preferably invasive breast carcinoma.

The term "sample from a patient" as used herein means any sample containing cancer cells that one wishes to detect including, but not limited to, biological fluids, tissue extracts, freshly harvested cells, and lysates of cells which have been incubated in cell cultures. In a preferred embodiment, the sample is breast tissue.

The term "control sample" includes any sample that can be used to establish a base or normal level, and may include tissue samples taken from healthy persons or samples mimicking physiological fluid.

The method of the invention may be used in the diagnosis and staging of cancer, in particular breast cancer. The invention may also be used to monitor the progression of a cancer and to monitor whether a particular treatment is effective or not. In particular, the method can be used to confirm the absence or removal of all tumor tissue following surgery, cancer chemotherapy, and/or radiation therapy. The methods can further be used to monitor cancer chemotherapy and tumor reappearance.

In an embodiment, the invention contemplates a method for monitoring the progression of cancer in a patient, comprising:

(a) obtaining a sample from a patient;

(b) determining the level of endoglycan expression in the sample;

(c) repeating steps (a) and (b) at a later point in time and comparing the result of step (b) with the result of step (c) wherein a difference in the level of endoglycan expression is indicative of the progression of the cancer in the patient.

In particular, decreased levels of endoglycan at the later time point may indicate that the cancer is progressing and that the treatment (if applicable) is not being effective. In contrast, increased levels of endoglycan at the later time point may indicate that the cancer is regressing and that the treatment (if applicable) is effective.

In a further embodiment, the invention contemplates a method for monitoring the progression of cancer in a patient, comprising:

(a) obtaining a sample from the patient;

(b) determining the level of endoglycan and podocalyxin in the sample; and

(c) repeating steps (a) and (b) at a later point in time and comparing the result of step (b) with the result of step (c) wherein a difference in the ratio of endoglycan to podocalyxin is indicative of the progression of the cancer in the patient.

The inventors have also shown that endoglycan is a marker of tumor metastasis. Accordingly, the present invention provides a method of determining whether or not a cancer is metastatic in a patient comprising:

(a) obtaining a sample from the patient;

(b) detecting the level of endoglycan in the sample; and

(c) comparing the level of endoglycan in the sample to a control sample, wherein decreased levels of endoglycan as compared to the control indicates that the cancer is metastatic.

In a further embodiment, the present invention provides a method of determining whether or not a cancer is metastatic in a patient comprising:

(a) obtaining a sample from the patient;

(b) detecting the level of endoglycan and podocalyxin in the sample; and

(c) comparing the ratio of endoglycan to podocalyxin in the sample to a control sample, wherein a decreased ratio of endoglycan to podocalyxin as compared to the control indicates that the cancer is metastatic.

A variety of methods can be employed for the above described diagnostic and prognostic evaluation of cancers involving endoglycan, and the identification of subjects with a predisposition to such disorders. Such methods may rely, for example, the detection of nucleic acid molecules encoding endoglycan (and podocalyxin), and fragments thereof, or the detection of the endoglycan protein (and podocalyxin protein) using, for example, antibodies directed against endoglycan (and podocalyxin), including peptide fragments. Each of these is described below.

(a) Methods for Detecting Nucleic Acid Molecules

In one embodiment, the methods of the invention involve the detection of nucleic acid molecules encoding endoglycan (and podocalyxin). Those skilled in the art can construct nucleotide probes for use in the detection of nucleic acid sequences encoding endoglycan (and podocalyxin) in samples. Suitable probes include nucleic acid molecules based on nucleic acid sequences encoding at least 5 sequential amino acids from regions of endoglycan (and podocalyxin), preferably they comprise 15 to 30 nucleotides. A nucleotide probe may be labeled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as ^{32}P, ^{3}H, ^{14}C or the like. Other detectable substances which may be used include antigens that are recognized by a specific labeled antibody, fluorescent compounds, enzymes, antibodies specific for a labeled antigen, and luminescent compounds. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization. Labeled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual (2nd ed.). The nucleic

acid probes may be used to detect genes, preferably in human cells, that encode endoglycan (and podocalyxin). The nucleotide probes may also be useful in the diagnosis of disorders involving an endoglycan in monitoring the progression of such disorders; or monitoring a therapeutic treatment. In an embodiment, the probes are used in the diagnosis of, and in monitoring the progression of cancer, preferably breast cancer.

The probe may be used in hybridization techniques to detect genes that encode endoglycan (and podocalyxin) proteins. The technique generally involves contacting and incubating nucleic acids (e.g. recombinant DNA molecules, cloned genes) obtained from a sample from a patient or other cellular source with a probe under conditions favorable for the specific annealing of the probes to complementary sequences in the nucleic acids. After incubation, the non-annealed nucleic acids are removed, and the presence of nucleic acids that have hybridized to the probe if any are detected.

The detection of nucleic acid molecules may involve the amplification of specific gene sequences using an amplification method such as polymerase chain reaction (PCR), followed by the analysis of the amplified molecules using techniques known to those skilled in the art. Suitable primers can be routinely designed by one of skill in the art.

Hybridization and amplification techniques described herein may be used to assay qualitative and quantitative aspects of expression of genes encoding endoglycan (and podocalyxin). For example, RNA may be isolated from a cell type or tissue known to express a gene encoding endoglycan (and podocalyxin), and tested utilizing the hybridization (e.g. standard Northern analyses) or PCR techniques referred to herein. The techniques may be used to detect differences in transcript size which may be due to normal or abnormal alternative splicing. The techniques may be used to detect quantitative differences between levels of full length and/or alternatively splice transcripts detected in normal individuals relative to those individuals exhibiting symptoms of a cancer involving an endoglycan protein or gene.

The primers and probes may be used in the above described methods *in situ* i.e. directly on tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections.

Accordingly, the present invention provides a method of detecting cancer in a patient comprising:

(a) obtaining a sample from the patient;

(b) extracting nucleic acid molecules comprising the endoglycan gene or portion thereof from the sample;

(c) amplifying the extracted nucleic acid molecules using the polymerase chain reaction;

(d) determining the presence of nucleic acid molecules encoding endoglycan; and

(e) comparing the level of endoglycan in the sample to a control sample, wherein decreased levels of endoglycan as compared to the control indicates that the patient has cancer.

The present invention also provides a method of detecting cancer in a patient comprising:

(a) obtaining a sample from the patient;

(b) extracting nucleic acid molecules comprising the endoglycan gene or portion thereof from the sample and the podocalyxin gene or portion thereof from the sample;

(c) amplifying the extracted nucleic acid molecules using the polymerase chain reaction;

(d) determining the presence of nucleic acid molecules encoding endoglycan and podocalyxin; and

(e) comparing the ratio of endoglycan to podocalyxin in the sample to a control sample, wherein a decreased ratio of endoglycan to podocalyxin as compared to the control indicates that the patient has cancer.

(b) Methods for Detecting Proteins

In another embodiment, the methods of the invention involve the detection of the endoglycan (and podocalyxin) protein. In one embodiment, the endoglycan protein is detected using antibodies that specifically bind to

endoglycan (and the podocalyxin protein is detected using antibodies that specifically bind to podocalyxin).

Antibodies to the endoglycan (and podocalyxin) may also be prepared using techniques known in the art. For example, by using a peptide of a endoglycan (or podocalyxin), polyclonal antisera or monoclonal antibodies can be made using standard methods. A mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the peptide which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the protein or peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g., the hybridoma technique originally developed by Kohler and Milstein (Nature 256, 495-497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., Immunol. Today 4, 72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. Monoclonal Antibodies in Cancer Therapy (1985) Allen R. Bliss, Inc., pages 77-96), and screening of combinatorial antibody libraries (Huse et al., Science 246, 1275 (1989)). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated.

The inventors have created a monoclonal antibody to endoglycan (Example 1). Accordingly, in another embodiment, the endoglycan protein is detected using a monoclonal antibody raised against a peptide of SEQ. ID. NO. 1 that specifically binds to endoglycan.

The term "specifically binds to endoglycan" means reactivity against endoglycan is clearly distinguishable from any reactivity against CD34 or podocalyxin.

The term "antibody" as used herein is intended to include fragments thereof which also specifically react with an endoglycan or fragments thereof (or a podocalyxin or fragments thereof). Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above. For example, F(ab')2 fragments can be generated by treating antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes the gene product of endoglycan antigens of the invention (See, for example, Morrison et al., Proc. Natl Acad. Sci. U.S.A. 81,6851 (1985); Takeda et al., Nature 314, 452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom patent GB 2177096B). It is expected that chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.

Monoclonal or chimeric antibodies specifically reactive with a protein of the invention as described herein can be further humanized by producing human constant region chimeras, in which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such immunoglobulin molecules may be made by techniques known in the art, (e.g., Teng et al., Proc. Natl. Acad. Sci. U.S.A., 80, 7308-7312

(1983); Kozbor et al., Immunology Today, 4, 7279 (1983); Olsson et al., Meth. Enzymol., 92, 3-16 (1982)), and PCT Publication WO92/06193 or EP 0239400). Humanized antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

Specific antibodies, or antibody fragments, such as, but not limited to, single-chain Fv monoclonal antibodies reactive against endoglycan (or podocalyxin) may also be generated by screening expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with peptides produced from the nucleic acid molecules of endoglycan. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries (See for example Ward et al., Nature 341, 544-546: (1989); Huse et al., Science 246, 1275-1281 (1989); and McCafferty et al. Nature, 348, 552-554 (1990)). Alternatively, a SCID-hu mouse, for example the model developed by Genpharm, can be used to produce antibodies or fragments thereof.

Antibodies specifically reactive with endoglycan (and podocalyxin), or derivatives, such as enzyme conjugates or labeled derivatives, may be used to detect endoglycan (and podocalyxin) in various samples (e.g. biological materials). They may be used as diagnostic or prognostic reagents and they may be used to detect abnormalities in the level of protein expression, or abnormalities in the structure, and/or temporal, tissue, cellular, or subcellular location of an endoglycan (and podocalyxin). *In vitro* immunoassays may also be used to assess or monitor the efficacy of particular therapies. The antibodies of the invention may also be used *in vitro* to determine the level of expression of a gene encoding endoglycan (and podocalyxin) in cells genetically engineered to produce an endoglycan (and podocalyxin) protein.

The antibodies may be used in any known immunoassays which rely on the binding interaction between an antigenic determinant of endoglycan (and podocalyxin) and the antibodies. Examples of such assays are radioimmunoassays, enzyme immunoassays (e.g. ELISA), immunofluorescence, immunoprecipitation, latex agglutination, hemagglutination, and histochemical tests. The antibodies may be used to

detect and quantify endoglycan (and podocalyxin) in a sample in order to determine its role in cancer and to diagnose the cancer.

In particular, the antibodies of the invention may be used in immunohistochemical analyses, for example, at the cellular and sub-subcellular level, to detect an endoglycan protein (and a podocalyxin protein), to localize it to particular cells and tissues, and to specific subcellular locations, and to quantitate the level of expression.

Cytochemical techniques known in the art for localizing antigens using light and electron microscopy may be used to detect endoglycan (and podocalyxin). Generally, an antibody of the invention may be labeled with a detectable substance and an endoglycan (and podocalyxin) protein may be localised in tissues and cells based upon the presence of the detectable substance. Examples of detectable substances include, but are not limited to, the following: radioisotopes (e.g., ^{3}H, ^{14}C, ^{35}S, ^{125}I, ^{131}I), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), luminescent labels such as luminol; enzymatic labels (e.g., horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase, acetylcholinesterase), biotinyl groups (which can be detected by marked avidin e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or calorimetric methods), predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached via spacer arms of various lengths to reduce potential steric hindrance. Antibodies may also be coupled to electron dense substances, such as ferritin or colloidal gold, which are readily visualised by electron microscopy.

The antibody or sample may be immobilized on a carrier or solid support which is capable of immobilizing cells, antibodies etc. For example, the carrier or support may be nitrocellulose, or glass, polyacrylamides, gabbros, and magnetite. The support material may have any possible configuration including spherical (e.g. bead), cylindrical (e.g. inside surface of a test tube or well, or the external surface of a rod), or flat (e.g. sheet, test

strip). Indirect methods may also be employed in which the primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity for the antibody reactive against endoglycan (and podocalyxin) protein. By way of example, if the antibody having specificity against endoglycan protein is a rabbit IgG antibody, the second antibody may be goat anti-rabbit gamma-globulin labeled with a detectable substance as described herein.

Where a radioactive label is used as a detectable substance, endoglycan (and podocalyxin) may be localized by radioautography. The results of radioautography may be quantitated by determining the density of particles in the radioautographs by various optical methods, or by counting the grains.

Labeled antibodies against endoglycan (and podocalyxin) protein may be used in locating tumor tissue in patients undergoing surgery i.e. in imaging. Typically for *in vivo* applications, antibodies are labeled with radioactive labels (e.g. iodine-123, iodine-125, iodine-131, gallium-67, technetium-99, and indium-111). Labeled antibody preparations may be administered to a patient intravenously in an appropriate carrier at a time several hours to four days before the tissue is imaged. During this period unbound fractions are cleared from the patient and the only remaining antibodies are those associated with tumor tissue. The presence of the isotope is detected using a suitable gamma camera. The labeled tissue can be correlated with known markers on the patient's body to pinpoint the location of the tumor for the surgeon.

Accordingly, in another embodiment the present invention provides a method for detecting cancer in a patient comprising:

(a) obtaining a sample from the patient;

(b) contacting the sample with an antibody that binds to endoglycan;

(c) detecting the level of endoglycan in the sample; and

(d) comparing the level of endoglycan in the sample to a control sample, wherein decreased levels of endoglycan as compared to the control indicates that the patient has cancer.

Accordingly, in another embodiment the present invention provides a method for detecting cancer in a patient comprising:

(a) obtaining a sample from the patient;

(b) contacting the sample with a first antibody that binds to endoglycan and a second antibody that binds to podocalyxin;

(c) detecting the level of endoglycan and podocalyxin in the sample; and

(d) comparing the ratio of endoglycan to podocalyxin in the sample to a control sample, wherein a decreased ratio of endoglycan to podocalyxin as compared to the control indicates that the patient has cancer.

In a specific embodiment of the invention, breast tissue samples can be screened using an anti-endoglycan antibody, such as the monoclonal antibody of Example 1 (and an anti-podocalyxin antibody). Antibody binding is detected using an appropriate detection system, preferably the Envision detection system, and staining is scored based on the intensity of cellular staining and the proportion of cells stained. Tissue samples are designated "0" (strong endoglycan staining in the majority of tumor cells, (and no discernable podocalyxin staining)), "1" (a mixture of weak and intense membrane staining for endoglycan (and podocalyxin)), "2" (weak endoglycan, (and strong podocalyxin), staining in the majority of tumor cells) or "3" (no discernable endoglycan staining, and (high podocalyxin staining)). Tissue samples exhibiting no discernable endoglycan staining in the majority of tumor cells (and high podocalyxin staining) (designated "3") have a significantly poorer outcome when compared with the other three designations.

II. Kits

The methods described herein may be performed by utilizing pre-packaged diagnostic kits comprising the necessary reagents to perform any of the methods of the invention. For example, the kits may include at least one specific nucleic acid or antibody described herein, which may be conveniently used, e.g., in clinical settings, to screen and diagnose patients and to screen and identify those individuals exhibiting a predisposition to developing cancer. The kits may also include nucleic acid primers for amplifying nucleic acids

encoding endoglycan (and podocalyxin) in the polymerase chain reaction. The kits can also include nucleotides, enzymes and buffers useful in the method of the invention as well as electrophoretic markers such as a 200 bp ladder. The kit will also include detailed instructions for carrying out the methods of the invention.

III. Therapeutic Methods

The finding by the present inventors that endoglycan is involved in tumor progression allows the development of therapies to treat cancer including the identification of compounds that modulate endoglycan. The present invention includes methods of treating cancer by modulating, preferably activating or stimulating, the levels of endoglycan on the cancer. The application also includes methods for the identification of compounds that modulate the biological activity of endoglycan that may be used for the treatment of cancers with decreased expression of endoglycan.

Accordingly, the present invention provides a method of modulating cancer cell growth by administering an effective amount of an agent that modulates endoglycan to a cell or animal in need thereof.

The terms "endoglycan" and "cancer" as used herein are as defined above in Section I.

The phrase "agent that modulates endoglycan" includes any agent that can stimulate or activate endoglycan (i.e. endoglycan agonists) as well as any agent that can inhibit or suppress endoglycan (i.e. endoglycan antagonists). Specific examples of endoglycan modulators are given below.

The phrase "modulate cancer cell growth" as used herein refers to the inhibition or suppression as well as the activation or stimulation of the formation, differentiation, growth or development of cancer cells.

The phrase "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired results (e.g. the modulation of cancer cell growth). Effective amounts of a molecule may vary according to factors such as the disease state, age, sex, weight of the animal. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be

administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The term "animal" as used herein includes all members of the animal kingdom which express endoglycan, preferably humans.

The term "a cell" includes a single cell as well as a plurality or population of cells. Administering an agent to a cell includes both *in vitro* and *in vivo* administrations.

In one aspect, the present invention provides a method of inhibiting cancer cell growth or treating cancer comprising administering an effective amount of endoglycan agonist to a cell or animal in need thereof.

The phrase "inhibiting cancer cell growth" means that the growth of the cancer cell is decreased or reduced as compared to the growth of the cancer cell in the absence of the endoglycan agonist.

The term "treatment or treating" as used herein means an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treating" can also mean prolonging survival as compared to expected survival if not receiving treatment.

In a preferred embodiment, the therapeutic methods of the invention are used to treat breast cancer.

The phrase "endoglycan agonist" means any agent that can activate or stimulate the activity, function or levels of expression of endoglycan on a cancer cell. Examples of endoglycan agonists include, but are not limited to, an antibody, small molecule, peptide mimetic, a nucleic acid encoding endoglycan or fragment thereof, or any molecule or protein that can antagonize podocalyxin on the surface of the tumor cell.

In one embodiment, the endoglycan agonist is a small molecule that binds to endoglycan. Accordingly, the present invention provides a method of

treating cancer comprising administering an effective amount of an agonist that can bind endoglycan to a cell or animal in need thereof.

The nucleic acids of the present invention may be ribonucleic or deoxyribonucleic acids and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The oligonucleotides may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

Other nucleic acids of the invention may contain modified phosphorous, oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. For example, the nucleic acid may contain phosphorothioates, phosphotriesters, methyl phosphonates, and phosphorodithioates. In an embodiment of the invention there are phosphorothioate bonds links between the four to six 3'-terminus bases. In another embodiment phosphorothioate bonds link all the nucleotides.

The nucleic acid of the invention may also comprise nucleotide analogs that may be better suited as therapeutic or experimental reagents. An example of a nucleotide analogue is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyamide backbone which is similar to that found in peptides (P.E. Nielsen, et al Science 1991, 254, 1497). PNA analogues have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. PNAs also bind stronger to a complimentary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other nucleic acids may contain nucleotides containing polymer backbones,

cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Patent No. 5,034,506). Nucleic acids may also contain groups such as reporter groups, a group for improving the pharmacokinetic properties of a nucleic acid, or a group for improving the pharmacodynamic properties of a nucleic acid. Nucleic acids may also have sugar mimetics.

The nucleic acids may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. The nucleic acids of the invention or a fragment thereof, may be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed with mRNA or the native gene e.g. phosphorothioate derivatives and acridine substituted nucleotides. The sequences may be produced biologically using an expression vector introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which sequences are produced under the control of a high efficiency regulatory region, the activity of which may be determined by the cell type into which the vector is introduced.

The nucleic acids may be introduced into tissues or cells using techniques in the art including vectors (retroviral vectors, adenoviral vectors and DNA virus vectors) or physical techniques such as microinjection. The nucleic acids may be directly administered *in vivo* or may be used to transfect cells *in vitro* which are then administered *in vivo*. In one embodiment, the nucleic acids may be delivered to macrophages and/or endothelial cells in a liposome formulation.

Peptide mimetics of endoglycan may also be prepared as endoglycan modulators or agonists. Such peptides may include competitive inhibitors, enhancers, peptide mimetics, and the like. All of these peptides as well as molecules substantially homologous, complementary or otherwise functionally or structurally equivalent to these peptides may be used for purposes of the present invention.

"Peptide mimetics" are structures which serve as substitutes for peptides in interactions between molecules (See Morgan et al (1989), Ann. Reports Med. Chem. 24:243-252 for a review). Peptide mimetics include synthetic structures which may or may not contain amino acids and/or peptide bonds but retain the structural and functional features of a peptide, or enhancer or inhibitor of the invention. Peptide mimetics also include peptoids, oligopeptoids (Simon et al (1972) Proc. Natl. Acad, Sci USA 89:9367); and peptide libraries containing peptides of a designed length representing all possible sequences of amino acids corresponding to an endoglycan peptide of the invention.

Peptide mimetics may be designed based on information obtained by systematic replacement of L-amino acids by D-amino acids, replacement of side chains with groups having different electronic properties, and by systematic replacement of peptide bonds with amide bond replacements. Local conformational constraints can also be introduced to determine conformational requirements for activity of a candidate peptide mimetic. The mimetics may include isosteric amide bonds, or D-amino acids to stabilize or promote reverse turn conformations and to help stabilize the molecule. Cyclic amino acid analogues may be used to constrain amino acid residues to particular conformational states. The mimetics can also include mimics of inhibitor peptide secondary structures. These structures can model the 3-dimensional orientation of amino acid residues into the known secondary conformations of proteins. Peptoids may also be used which are oligomers of N-substituted amino acids and can be used as motifs for the generation of chemically diverse libraries of novel molecules.

Peptides derived from endoglycan isoforms may also be used to identify lead compounds for drug development. The structure of the peptides described herein can be readily determined by a number of methods such as NMR and X-ray crystallography. A comparison of the structures of peptides similar in sequence, but differing in the biological activities they elicit in target molecules can provide information about the structure-activity relationship of the target. Information obtained from the examination of structure-activity

relationships can be used to design either modified peptides, or other small molecules or lead compounds that can be tested for predicted properties as related to the target molecule. The activity of the lead compounds can be evaluated using assays similar to those described herein.

Information about structure-activity relationships may also be obtained from co-crystallization studies. In these studies, a peptide with a desired activity is crystallized in association with a target molecule, and the X-ray structure of the complex is determined. The structure can then be compared to the structure of the target molecule in its native state, and information from such a comparison may be used to design compounds expected to possess the desired activity. Accordingly, in one embodiment, endoglycan may be cocrystallized with podocalyxin and the structure can then be compared to the structure of podocalyxin in its native state, to obtain information that may be used to design compounds that mimic endoglycan antagonism of podocalyxin.

IV. Screening Assays

The present invention also includes screening assays for identifying agents that modulate endoglycan and that are useful in modulating cancer cell growth. Agents that modulate include agents that stimulate endoglycan (endoglycan agonists) and agents that inhibit endoglycan (endoglycan antagonists).

In accordance with one embodiment, the invention provides a method for screening candidate compounds for their ability to modulate the activity of endoglycan. The method comprises providing an assay system for assaying endoglycan levels, assaying the levels in the presence or absence of the candidate or test compound and determining whether the compound has increased or decreased endoglycan levels.

Accordingly, the present invention provides a method for identifying a compound that modulates endoglycan comprising:

(a) incubating a test compound with endoglycan or a nucleic acid encoding endoglycan; and

(b) determining the effect of the compound on endoglycan activity or expression and comparing with a control (i.e. in the absence of the

test substance), wherein a change in the endoglycan activity or expression as compared to the control indicates that the test compound modulates endoglycan.

In one embodiment, the screening assay can be used to identify endoglycan agonists.

Accordingly, the present invention provides a screening assay for identifying an agonist of endoglycan comprising the steps of:

(a) incubating a test substance with endoglycan; and

(b) determining whether or not the test substance activates endoglycan activity, function or expression levels.

The endoglycan is generally immobilized in the above assays. Preferably, the endoglycan is expressed on the surface of a cell, more preferably a cancer cell.

Since endoglycan and podocalyxin both bind to NHERF, the invention also provides a method for identifying a compound that modulates NHERF comprising:

(a) incubating a test compound with NHERF or with cells expressing NHERF on its surface; and

(b) determining the effect of the compound on NHERF activity or expression and comparing with a control (i.e. in the absence of the test substance), wherein a change in the NHERF activity or expression as compared to the control indicates that the test compound modulates NHERF. A change in NHERF activity may include a change in response to endoglycan or podocalyxin.

Agents that modulate include agents that stimulate NHERF (NHERF agonists) and agents that inhibit NHERF (NHERF antagonists). In one embodiment, the screening assay can be used to identify NHERF antagonists.

In all of the above screening assays, the test compound can be any compound which one wishes to test including, but not limited to, proteins, peptides, nucleic acids (including RNA, DNA, antisense oligonucleotides, peptide nucleic acids), carbohydrates, organic compounds, small molecules,

natural products, library extracts, bodily fluids and other samples that one wishes to test for modulators of endoglycan or NHERF.

One skilled in the art will appreciate that many methods can be used in order to determine whether or not a test substance can activate endoglycan or modulate NHERF and therefore inhibit cancer cell growth. Once a compound is identified in a screening assay (Endoglycan agonist or NHERF modulator), it may be tested in *in vitro* or *in vivo* assays to determine its effect on cancer cell growth.

The screening methods of the invention include high-throughput screening applications. For example, a high-throughput screening assay may be used which comprises any of the methods according to the invention wherein aliquots of cells transfected with endoglycan are exposed to a plurality of test compounds within different wells of a multi-well plate. Further, a high-throughput screening assay according to the invention involves aliquots of transfected cells which are exposed to a plurality of candidate factors in a miniaturized assay system of any kind. Another embodiment of a high-throughput screening assay could involve exposing a transfected cell population simultaneously to a plurality of test compounds.

The method of the invention may be "miniaturized" in an assay system through any acceptable method of miniaturization, including but not limited to multi-well plates, such as 24, 48, 96 or 384-wells per plate, micro-chips or slides. The assay may be reduced in size to be conducted on a micro-chip support, advantageously involving smaller amounts of reagent and other materials. Any miniaturization of the process which is conducive to high-throughput screening is within the scope of the invention.

The invention extends to any compounds or modulators of endoglycan identified using the screening method of the invention that are useful in treating cancer.

The invention also includes a pharmaceutical composition comprising a modulator of endoglycan identified using the screening method of the invention in admixture with a suitable diluent or carrier. The invention further includes a method of preparing a pharmaceutical composition for use in

modulating cancer cell growth comprising mixing a modulator of endoglycan identified according to the screening assay of the invention with a suitable diluent or carrier.

The present invention also includes all business applications of the screening assay of the invention including conducting a drug discovery business. Accordingly, the present invention also provides a method of conducting a drug discovery business comprising:

(a) providing one or more assay systems for identifying a modulator of endoglycan;

(b) conducting therapeutic profiling of modulators identified in step (a), or further analogs thereof, for efficacy and toxicity in animals; and

(c) formulating a pharmaceutical preparation including one or more modulators identified in step (b) as having an acceptable therapeutic profile.

In certain embodiments, the subject method can also include a step of establishing a distribution system for distributing the pharmaceutical preparation for sale, and may optionally include establishing a sales group for marketing the pharmaceutical preparation.

The present invention also provides a method of conducting a target discovery business comprising:

(a) providing one or more assay systems for identifying modulators of endoglycan;

(b) (optionally) conducting therapeutic profiling of modulators identified in step (a) for efficacy and toxicity in animals; and

(c) licensing, to a third party, the rights for further drug development and/or sales for modulators identified in step (a), or analogs thereof.

V. Pharmaceutical Compositions

The present invention includes pharmaceutical compositions containing one or more modulators of endoglycan. Accordingly, the present invention provides a pharmaceutical composition for use in modulating cancer cell

growth comprising an effective amount of endoglycan modulator in admixture with a suitable diluent or carrier.

In one embodiment, the present invention provides a pharmaceutical composition for use in treating cancer comprising an effective amount of a endoglycan agonist in admixture with a suitable diluent or carrier.

Such pharmaceutical compositions can be for intralesional, intravenous, topical, rectal, parenteral, local, inhalant or subcutaneous, intradermal, intramuscular, intrathecal, transperitoneal, oral, and intracerebral use. The composition can be in liquid, solid or semisolid form, for example pills, tablets, creams, gelatin capsules, capsules, suppositories, soft gelatin capsules, gels, membranes, tubelets, solutions or suspensions. The endoglycan or ligand is preferably injected in a saline solution either intravenously, intraperitoneally or subcutaneously.

The pharmaceutical compositions of the invention can be intended for administration to humans or animals. Dosages to be administered depend on individual needs, on the desired effect and on the chosen route of administration.

The pharmaceutical compositions can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985).

On this basis, the pharmaceutical compositions include, albeit not exclusively, the active compound or substance in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids. The pharmaceutical compositions may additionally contain other anti-cancer agents.

A pharmaceutical composition comprising the nucleic acid molecules of the invention may be used in gene therapy to treat cancer. Recombinant

molecules comprising a nucleic acid sequence encoding endoglycan molecule of the invention, or fragment thereof, may be directly introduced into cells or tissues *in vivo* using delivery vehicles such as retroviral vectors, adenoviral vectors and DNA virus vectors. They may also be introduced into cells *in vivo* using physical techniques such as microinjection and electroporation or chemical methods such as coprecipitation and incorporation of DNA into liposomes. Recombinant molecules may also be delivered in the form of an aerosol or by lavage. The nucleic acid molecules of the invention may also be applied extracellularly such as by direct injection into cells.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1

Tissue Distribution of CD34 Family Members

Data was compiled from published analyses on human and mouse CD34, Podocalyxin and Endoglycan (Krause 1996, McNagny 1997, Doyonnas 2001, Sassetti 2000) and from our unpublished observations on mouse Endoglycan. Endoglycan and Podocalyxin expression profiles were generated using unpublished data obtained from: 1) Northern blots of hematopoietic lineage cell lines, 2) RT-PCR of sorted hematopoietic subsets from bone marrow, 3) antibody stains and flow cytometry analysis using existing antibodies to CD34 (RAM34) Podocalyxin (PCLP1) and 4) Immunohistochemistry using the same antibodies. Results are shown in Table 1.

Preparation of Monoclonal Antibody with specific binding against Endoglycan

To make the rat monoclonal antibody, rats were immunized with a peptide corresponding to sequence from the extracellular domain: **V A S M E D P G Q A P D L P N L P S I L P K M D L A E P P W H M P L Q G G C** linked to KLH and boosted with the entire extracellular domain

fused to the Fc portion of Rabbit IgG1. Hybridomas were made using standard protocols and antibodies from these hybridomas were screened for reactivity with the peptide and Fc-fusion protein by ELISA. They were also screened for the ability to stain a rat myeloma cell line, Y3, which had been transfected to express full length Endoglycan. One antibody passed all criteria (F4B10). This antibody did not react with Y3 cells expressing CD34 or Podocalyxin so the antibody is specific for Endoglycan and not related family members (Figure 3). In addition, this antibody reacts with mouse and human Endoglycan and so it may be a useful reagent for both species.

Expression of Endoglycan in relation to Podocalyxin

Endoglycan and Podocalyxin have a mirror image pattern of expression in breast cancer cell lines (Figure 4). In MDA-231: metastatic tumor line where cells are non-polarized, Podocalyxin expression is high, whereas Endoglycan expression is negative. In MCF-7, a relatively non-metastatic line, cells maintain normal polarity, Podocalyxin expression is low, whereas Endoglycan is highly expressed. In T47D: a relatively non-metastatic line, cells maintain normal polarity, Podocalyxin expression is low, whereas Endoglycan expression is high. This was determined by indirect immunofluorescence using our new antibody and flow cytometry (FACS).

Function of Endoglycan:

Despite Endoglycan's similarity to CD34 and Podocalyxin, it may have a different function. Endoglycan was expressed in CD34/CD43 deficient mast cells. Pure mast cell cultures can be obtained by culturing mouse bone marrow in IL-3 for > 4 weeks. Although normal mast cells grow in single cell suspensions, mast cells grown from CD34/CD43 KO mice tend to form large aggregates. Infection of mast cells with a retrovirus expressing ectopic CD34 reverses this aggregation and suggests that the normal function of CD34 is to block adhesion. In side by side experiments, ectopic expression of Endoglycan had no effect suggesting that it does not block adhesion and may instead have a pro-adhesive function. (Figure 5).

Since Endoglycan and Podocalyxin have very similar sequences in the cytoplasmic domain, they may be natural antagonists of each other: Endoglycan may promote adhesion, maintain cell polarity, and block metastasis, and Podocalyxin may block adhesion and decrease cell polarity and increase metastasis. One theory is that endoglycan and podocalyxin compete for binding to NHERF1; a molecule that has previously been shown to link Podocalyxin to the the actin cytoskeleton (Takeda et al., 2001). This then would allow these molecules (with opposing functions) to compete for localization in adhesion structures.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Table 1:Tissu Distribution of CD34 Family Members

Tissue/Cells	Endoglycan	Podocalyxin	CD34
Multipotent hematopoetic precursors			
Adult	+	+	+
Embryo	+	+	+
Monopotent precursors			
Erythroid	+	+	-
Thrombocytic	?	+	+
Myeloid	+/-	-	+
Lymphoid (subset of thymocytes)	+?	+	+
Mature hematopoetic cells			
B Cells (LPS activated)	+	-	-
T Cells	-	-	-
Macrophages	-	-	-
Granulocytes	-	-	-
Eosinophils	-	-	-
Mast Cells	-	-	+
Erythrocytes	+*	+*	-
Platelets	?	+	-
Vessels			
Vascular endothelial	-	+	+
Vascular smooth muscle	+	-	-
Intestinal Epithelial	+	-	-
Podocytes	+/-	+	-
Brain (Neurons)	+	+**	?
Boundary Elements(mesothelial)	-	+	-

* embryonic erythrocytes only
**ependymal layer only

FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

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WE CLAIM:

1. A method of detecting cancer in a patient comprising:

(a) obtaining a sample from the patient;

(b) determining the level of endoglycan in the sample; and

(c) comparing the level of endoglycan in the sample to a control sample, wherein decreased levels of endoglycan as compared to the control indicates that the patient has cancer.

2. A method according to claim 1 wherein the cancer is breast cancer.

3. A method according to claim 1 wherein the level of nucleic acid molecules encoding endoglycan are determined in step (b).

4. A method according to claim 3 wherein the level of expression of endoglycan mRNA is determined.

5. A method according to claim 1 wherein the level of endoglycan protein is determined in step (b).

6. A method according to claim 5 wherein an antibody is used to determine the levels of the endoglycan protein.

7. A method of detecting cancer in a patient comprising:

(a) obtaining a sample from the patient;

(b) determining the level of endoglycan and podocalyxin in the sample; and

(c) comparing the ratio of endoglycan to podocalyxin in the sample to a control sample, wherein a decreased ratio as compared to the control indicates that the patient has cancer.

8. A method according to claim 7 wherein the cancer is breast cancer.

9. A method according to claim 7 wherein the level of nucleic acid molecules encoding endoglycan and podocalyxin are determined in step (b).

10. A method according to claim 9 wherein the levels of expression of endoglycan mRNA and podocalyxin mRNA are determined.

11. A method according to claim 7 wherein the levels of endoglycan protein and podocalyxin protein are determined in step (b).

12. A method according to claim 11 wherein antibodies are used to determine the levels of the endoglycan protein and the podocalyxin protein.

13. A method of monitoring the progression of cancer in a patient comprising:

(a) obtaining a sample from the patient;

(b) determining the level of endoglycan in the sample;

(c) repeating steps (a) and (b) at a later point in time and comparing the result of step (b) with the result of step (c) wherein a difference in the level of endoglycan is indicative of the progression of the cancer in the patient.

14. A method according to claim 13 wherein the cancer is breast cancer.

15. A method according to claim 13 wherein the level of nucleic acid molecules encoding endoglycan are determined in step (b).

16. A method according to claim 15 wherein the level of expression of endoglycan mRNA is determined.

17. A method according to claim 13 wherein the level of endoglycan protein is determined in step (b).

18. A method according to claim 17 wherein an antibody is used to determine the levels of the endoglycan protein.

19. A method of monitoring the progression of cancer in a patient comprising:

(a) obtaining a sample from the patient;

(b) determining the level of endoglycan and podocalyxin in the sample;

(c) repeating steps (a) and (b) at a later point in time and comparing the result of step (b) with the result of step (c) wherein a difference in the ratio of endoglycan to podocalyxin is indicative of the progression of the cancer in the patient.

20. A method according to claim 19 wherein the cancer is breast cancer.

21. A method according to claim 19 wherein the level of nucleic acid molecules encoding endoglycan and podocalyxin are determined in step (b).

22. A method according to claim 21 wherein the levels of expression of endoglycan mRNA and podocalyxin mRNA are determined.

23. A method according to claim 19 wherein the levels of endoglycan protein and podocalyxin protein are determined in step (b).

24. A method according to claim 23 wherein antibodies are used to determine the levels of the endoglycan protein and the podocalyxin protein.

25. A method of determining whether or not a cancer is metastatic in a patient comprising:

(a) obtaining a sample from the patient;

(b) detecting the level of endoglycan in the sample; and

(c) comparing the level of endoglycan in the sample to a control sample, wherein a decreased level of endoglycan as compared to the control indicates that the cancer is metastatic.

26. A method according to claim 25 wherein the cancer is breast cancer.

27. A method according to claim 25 wherein the level of nucleic acid molecules encoding endoglycan are determined in step (b).

28. A method according to claim 27 wherein the level of expression of endoglycan mRNA is determined.

29. A method according to claim 25 wherein the level of endoglycan protein is determined in step (b).

30. A method according to claim 29 wherein an antibody is used to determine the levels of the endoglycan protein.

31. A method of determining whether or not a cancer is metastatic in a patient comprising:

(a) obtaining a sample from the patient;

(b) detecting the level of endoglycan and podocalyxin in the sample; and

(c) comparing the ratio of endoglycan to podocalyxin in the sample to a control sample, wherein a decreased ratio of endoglycan to podocalyxin as compared to the control indicates that the cancer is metastatic.

32. A method according to claim 31 wherein the cancer is breast cancer.

33. A method according to claim 31 wherein the level of nucleic acid molecules encoding endoglycan and podocalyxin are determined in step (b).

34. A method according to claim 33 wherein the levels of expression of endoglycan mRNA and podocalyxin mRNA are determined.

35. A method according to claim 31 wherein the levels of endoglycan protein and podocalyxin protein are determined in step (b).

36. A method according to claim 35 wherein antibodies are used to determine the levels of the endoglycan protein and the podocalyxin protein.

37. A kit for detecting cancer in a patient comprising (i) reagents for conducting a method according to claim 1 and (ii) instructions for its use.

38. A kit according to claim 37 wherein the reagents comprise nucleic acid primers for amplifying mRNA coding for endoglycan in a reverse transcriptase polymerase chain reaction.

39. A kit according to claim 37 wherein the reagents comprise antibodies specific to the endoglycan protein.

40. A kit for detecting cancer in a patient comprising (i) reagents for conducting a method according to claim 7 and (ii) instructions for its use.

41. A kit according to claim 40 wherein the reagents comprise nucleic acid primers for amplifying mRNA coding for endoglycan and podocalyxin in a reverse transcriptase polymerase chain reaction.

42. A kit according to claim 40 wherein the reagents comprise antibodies specific to the endoglycan protein and the podocalyxin protein.

43. A kit for monitoring the progression of cancer in a patient comprising (i) reagents for conducting a method according to claim 13 and (ii) instructions for its use.

44. A kit according to claim 43 wherein the reagents comprise nucleic acid primers for amplifying mRNA coding for endoglycan in a reverse transcriptase polymerase chain reaction.

45. A kit according to claim 43 wherein the reagents comprise antibodies specific to the endoglycan protein.

46. A kit for monitoring the progression of cancer in a patient comprising (i) reagents for conducting a method according to claim 19 and (ii) instructions for its use.

47. A kit according to claim 46 wherein the reagents comprise nucleic acid primers for amplifying mRNA coding for endoglycan and podocalyxin in a reverse transcriptase polymerase chain reaction.

48. A kit according to claim 46 wherein the reagents comprise antibodies specific to the endoglycan protein and the podocalyxin protein.

49. A kit for determining whether or not a cancer is metastatic in a patient comprising (i) reagents for conducting a method according to claim 25 and (ii) instructions for its use.

50. A kit according to claim 49 wherein the reagents comprise nucleic acid primers for amplifying mRNA coding for endoglycan in a reverse transcriptase polymerase chain reaction.

51. A kit according to claim 49 wherein the reagents comprise antibodies specific to endoglycan protein.

52. A kit for determining whether or not a cancer is metastatic in a patient comprising (i) reagents for conducting a method according to claim 31 and (ii) instructions for its use.

53. A kit according to claim 52 wherein the reagents comprise nucleic acid primers for amplifying mRNA coding for endoglycan and podocalyxin in a reverse transcriptase polymerase chain reaction.

54. A kit according to claim 52 wherein the reagents comprise antibodies specific to the endoglycan protein and the podocalyxin protein.

55. A method of modulating cancer cell growth by administering an effective amount of an agent that modulates endoglycan to a cell or animal in need thereof.

56. A method of inhibiting cancer cell growth or treating cancer comprising administering an effective amount of endoglycan agonist to a cell or animal in need thereof.

57. A method according to claim 29 wherein the endoglycan agonist is a nucleic acid encoding endoglycan or a fragment thereof.

58. A method according to claim 29 or 30 wherein the cancer is breast cancer.

59. A method for identifying a compound that modulates endoglycan comprising:

(a) incubating a test compound with endoglycan or a nucleic acid encoding endoglycan; and

(b) determining the effect of the compound on endoglycan activity or expression and comparing with a control, wherein a change in the endoglycan

activity or expression as compared to the control indicates that the test compound modulates endoglycan.

60. A screening assay for identifying an agonist of endoglycan comprising the steps of:

(a) incubating a test substance with endoglycan; and

(b) determining whether or not the test substance inhibits endoglycan activity, function or expression levels.

61. A pharmaceutical composition for use in modulating cancer cell growth comprising an effective amount of endoglycan modulator in admixture with a suitable diluent or carrier.

62. A pharmaceutical composition for use in treating cancer comprising an effective amount of an endoglycan agonist in admixture with a suitable diluent or carrier.

ABSTRACT OF THE DISCLOSURE

Methods and kits for detecting cancer and monitoring cancer progression are described. The method involves analyzing a sample containing nucleic acids or proteins from a patient for decreased expression of endoglycan.

Fig. 1: Genomic loci, motifs and splicing

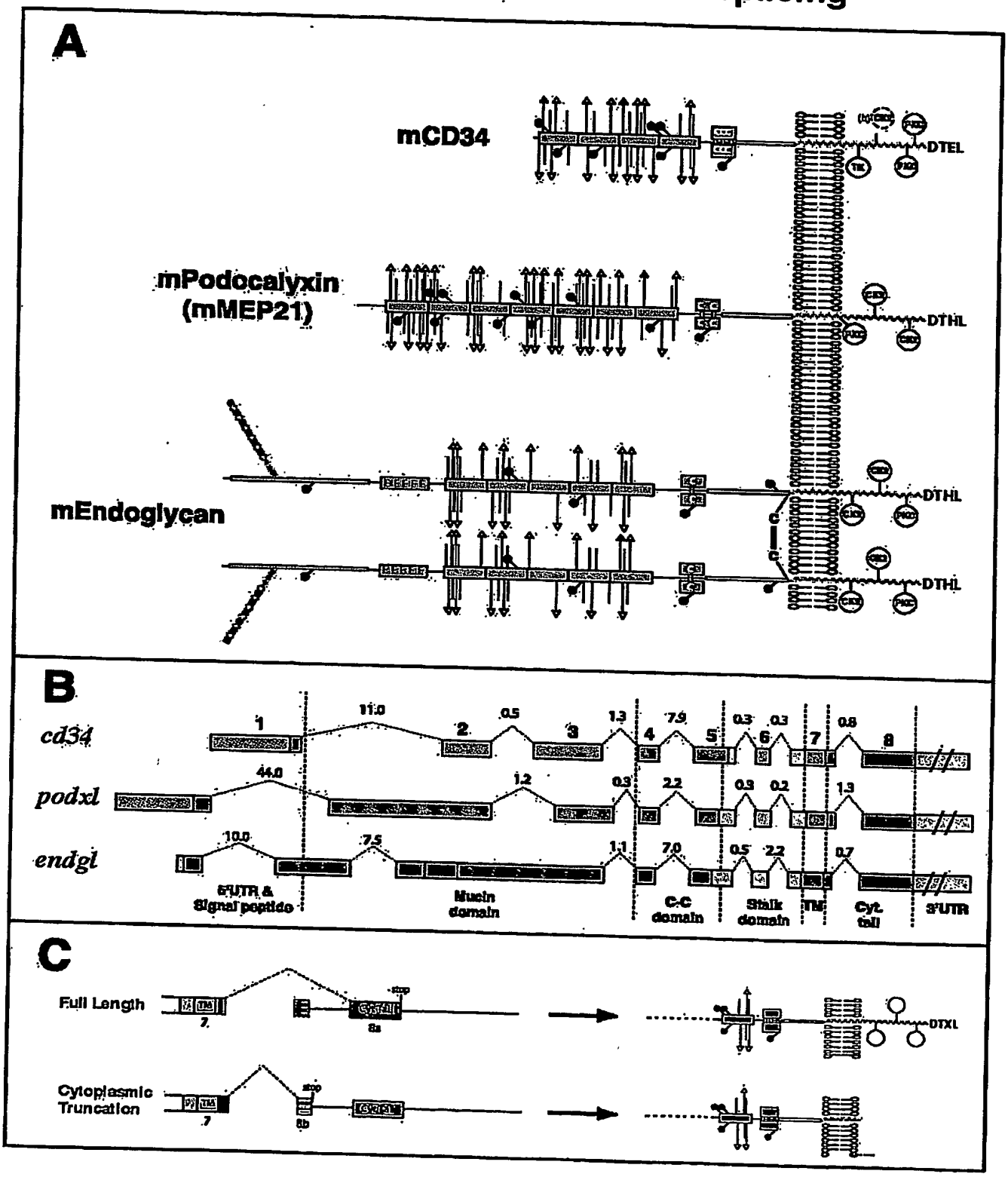
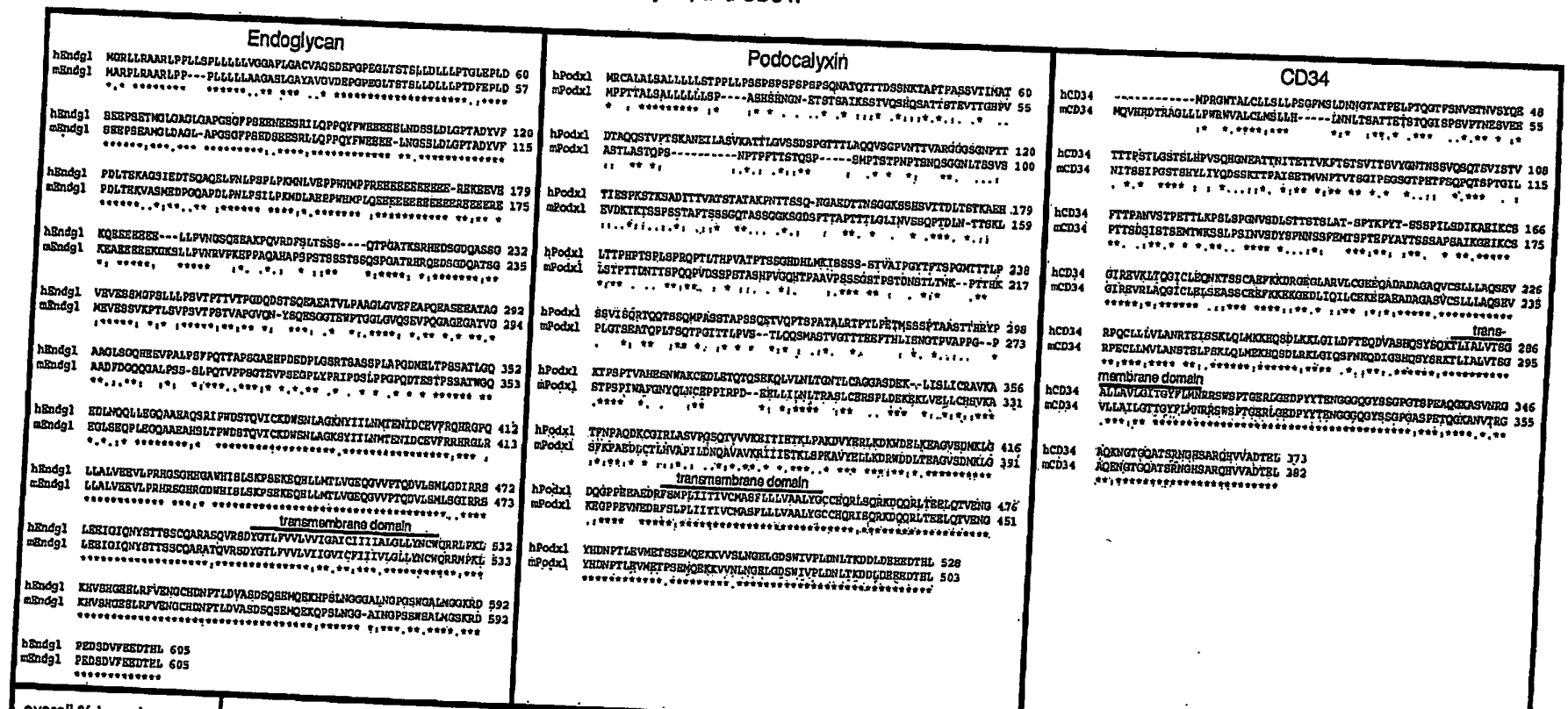


Fig. 2: homologies

Amino acid alignment of mouse and human Endoglycan, Podocalyxin, and CD34:



	Endoglycan	Podocalyxin	CD34
overall % homology:	76%	43%	65%
extracellular % homology:	72%	30%	55%
cytoplasmic % homology:	92%	96%	93%

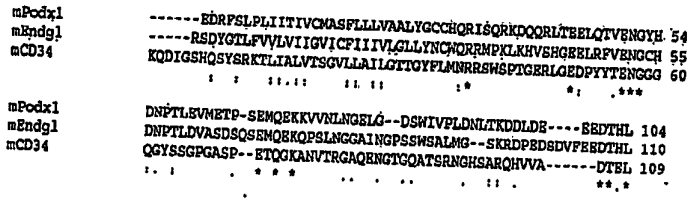


Fig.3

The anti-mEndoglycan Antibody is Specific for Endoglycan

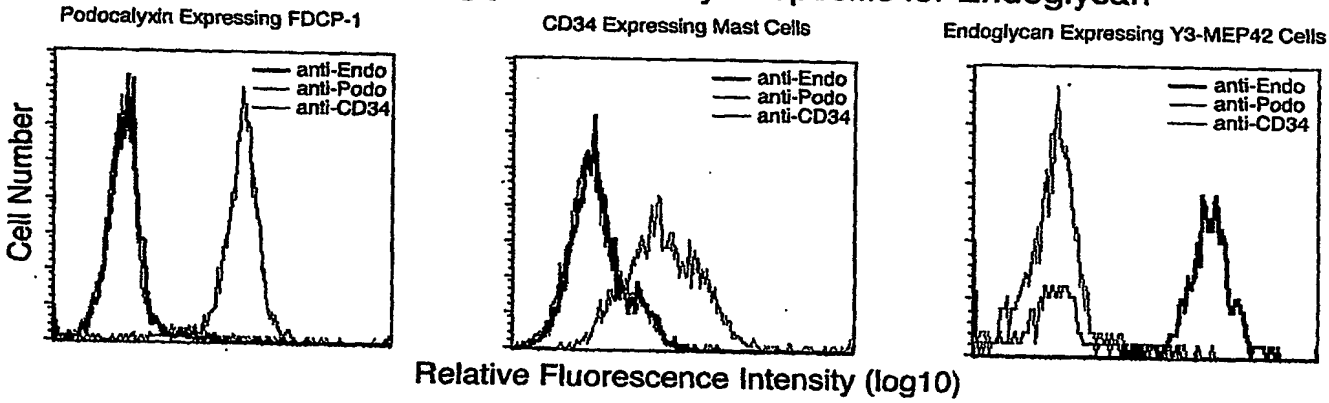


Fig. 4

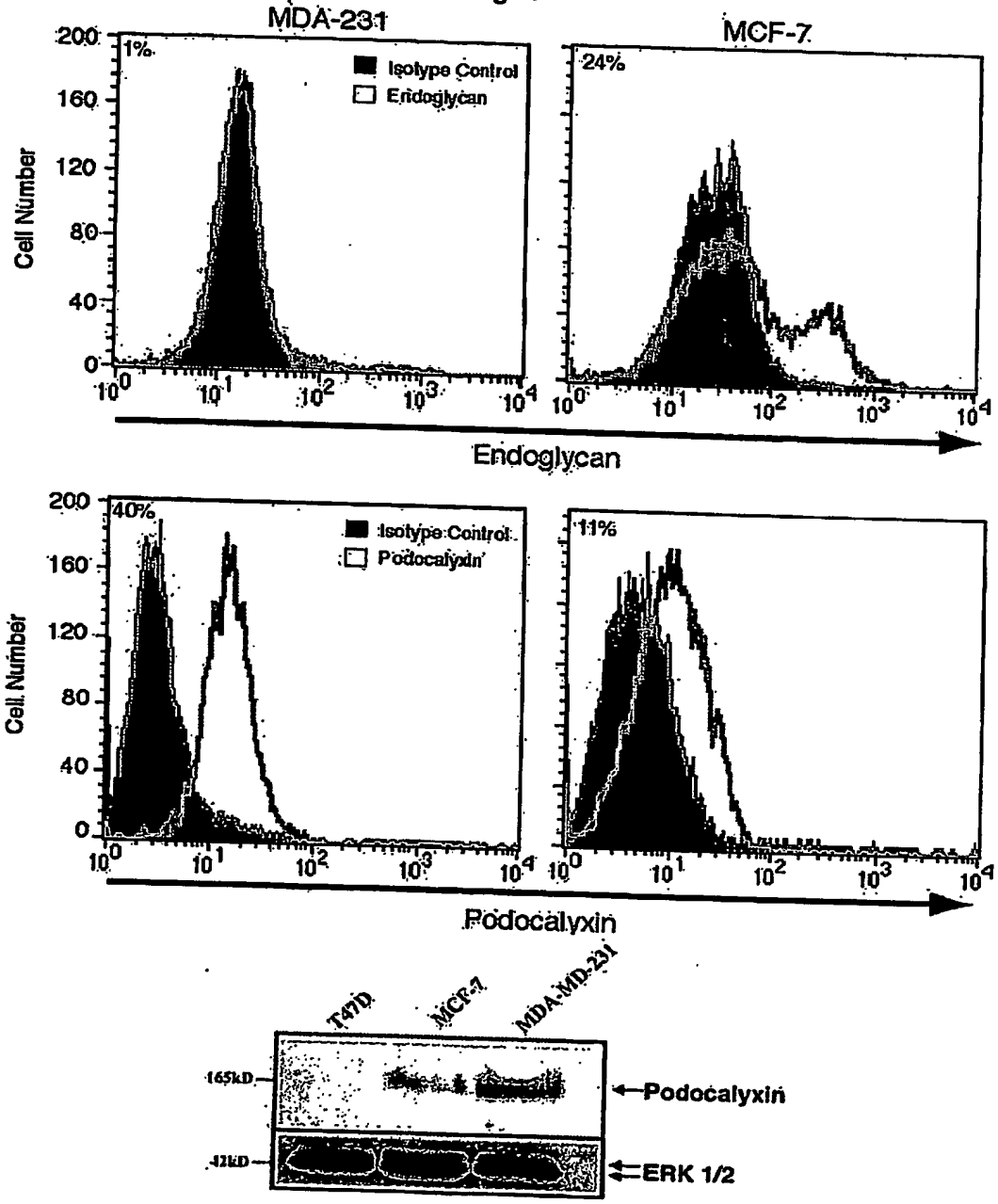


Fig. 5: Failure of Endoglycan to Inhibit mast cell aggregation